COMPLEXITY OF TOXINS FROM *CLOSTRIDIUM SEPTICUM* AND *CLOSTRIDIUM CHAUVOEI*

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Production of toxins by *Clostridium septicum* and *Clostridium chauwoei* has been the subject of many conflicting reports. Roux and Chamberland (1887) first noted that 3 to 4 day filtrates were antigenic; conferring on guinea pigs an immunity against infection with viable organisms. Production of a lethal toxin by *C. septicum* was however, demonstrated by Leclainche (1898) and Leclainche and Morel (1901), and for *C. chauwoei* by Duenschmann (1894) and Leclainche and Vallée (1900). This was later questioned by a number of workers, (Barger and Dale, 1915; O'Brien, 1929). Kojima (1923) divided the strains of *C. chauwoei* he tested into two groups: one nontoxic and the other toxic, the latter producing *in vitro* lethal toxin which was neutralized by *C. septicum* sera. Leclainche and Vallée (1923) advocated examination for two toxins: an exotoxin which appeared in culture filtrates after 24 to 48 hr of incubation and an endotoxin produced on aging and lysis of these cultures. Similar observations were also made by Berg (1923) who obtained maximum toxin-production of a *C. chauwoei* strain after 30 days of incubation.

It is, however, clear that filtrates of the two organisms comprise more than one toxic substance. Thus in addition to the *C. septicum* lethal toxin, Eisenberg, as early as 1907, described an oxygen-labile hemolysin which he found associated with a leucocidin. This has been recently confirmed by Oakley and Warrack (1951) and Warrack *et al.* (1951) who found it antigenically distinct from the deoxyribonuclease of *Clostridium perfringens* (*C. welchii*) type A and considered it as *C. septicum* β-toxin.

In attempting to study the toxins of *C. septicum* and *C. chauwoei* and to elucidate some of the relationships between their components, a number of toxigenic strains was investigated under different cultural and environmental conditions. The complexity of the toxins was established and a number of components was identified.

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MATERIALS AND METHODS

Organisms. The main part of this work was done using 8 strains which showed, on preliminary testing, some promising results. As they were kept in the laboratory for some time, their virulence was enhanced by inoculating them into guinea pigs. These were the *C. septicum* strains 281, 501 (N.C.T.C., London) Amatsi and Fenten (Mlle. Guillaumie, L'Institut Pasteur, Paris) and the *C. chauwoei* strains 8070 (N.C.T.C., London), 1500 (Wellcome Research Laboratories, Kent), Vallée (Mlle. Guillaumie, L'Institut Pasteur, Paris), and Chau 2 (Dr. Raynaud, L'Institut Pasteur, Garches, France).

Sera. These were kindly supplied by Professor C. L. Oakley and comprised the *C. septicum* antitoxic sera, EX 586, EX 588, EX 928, EX 1632, and Prévot 1000; the *C. chauwoei* antitoxic sera EX 1370, EX 1658, EX 1664, and Prévot 46942; and the anti-streptolysin-O serum, RX 5619. Normal horse sera (and sodium deoxyribonucleate) were supplied by the Wellcome Research Laboratories.

Media. Cultures were grown in 1 per cent glucose broth and in chopped meat broth containing 1 per cent glucose (pH about 7.6) with or without 5 per cent normal horse serum. Incubation at 37 C usually lasted for 18 to 24 hr. After centrifugation at 3000 rpm (and 10 C) for 30 min, the supernatant fluids were filtered through a Chamberland filter and stored at 4 C.

Hemolysin and necrotoxin assay. Filtrates intended for use in oxygen-labile hemolysin tests were placed with 0.2 per cent sodium hydrosulfite in screw-capped bottles and stored at 4 C for at least 2 weeks (Todd, 1938). On the other hand, filtrates used for their oxygen-stable hemolysin or their necrotoxin showed on storage rapid deterioration of their toxin content and were used as fresh as possible.

Varying amounts of the filtrates, first in doubling dilutions, then in 10 per cent differences, were mixed in 0.5 ml amounts with one arbitrary unit of the standard antiserum. After allowing the tubes to stand for ½ hr at room temperature, 1 ml of 2 per cent washed sheep erythrocytes was added, and tubes shaken and incubated in a water bath at 37 C for 2 hr. The last tube showing complete hemolysis in the 10 per cent series indicated the test dose of toxin which was then used to assay *C. septicum* and *C. chauvoei* antitoxin as well as anti-streptolysin-O and various normal sera.

However, with the necrotoxin (as well as the oxygen-stable hemolysin) assay would only be made in doubling dilution series. Two-tenths ml of each dilution was injected intracutaneously into the depilated flanks of albino guinea pigs. The lowest dilution of the filtrate shown to be inhibited after 24 hr by 1 unit of the standard serum was considered to contain one test dose. At least 2 such doses were used to assay the different sera and results of the doubling dilution assay, considered after 48 hr, were taken as final.

Hyaluronidase and deoxyribonuclease assay. These were made in the manner described by Oakley and Warrack (1951) and Warrack *et al.* (1951), using ox-synovial fluid and sodium deoxyribonucleate. Assay of sera for their antihyaluronidase or antideoxyribonuclease content was made, as in the assay for the oxygen-labile hemolysin, by identifying, in doubling dilution series and then in 10 per cent differences, the minimum amount of filtrate which, when mixed with 1 unit of the standard antitoxic serum, just produced a good spread in the ACRA (acid-congo red-alcohol) test. This test dose of the filtrate was then used to assay other antitoxic sera.

RESULTS

Production of the oxygen-labile hemolysin was demonstrated in glucose broth filtrates of most of the strains of *C. septicum* and *C. chauwoei* examined. The addition of chopped meat did not directly affect its production, but ensured a vigorous growth, whereas the addition of 5 per cent normal horse serum to the growing culture medium seemed to favor the production of the

TABLE 1

*Effect of pH, and of saline and phosphate buffer diluents, on oxygen-labile hemolysin titer**

Filtrate	pH	Vol of Filtrate					
		0.50	0.25	0.125	0.06	0.03	0.01
Clostridium chauvoei strain Chau 2:							
Unadjusted	5.6	+++	+++	+++	+++	+++	++
Adjusted	6.0	+++	+++	+++	+++	++	−
	6.5	+++	+++	+++	++	−	
	7.0	+++	+++	++	−	−	−
	7.5	+++	+++	++	−	−	−
Saline diluent		+++	+++	+++	+++	+++	++
Phosphate buffer Diluent	6.0	+++	+++	+++	++	−	−
	7.0	+++	+++	++	+	−	−
Clostridium septicum strain Amatsi:							
Unadjusted	5.4	+++	+++	+++	+++	−	−
Adjusted	6.0	+++	+++	++	−	−	−
	6.5	+++	+++	+	−	−	−
	7.0	+++	+++	+	−	−	−
	7.5	+++	+++	−	−	−	−
Saline diluent		+++	+++	+++	+++	−	−
Phosphate buffer Diluent	6.0	+++	+++	++	−	−	−
	7.0	+++	+++	++	−	−	−

* +++ = complete hemolysis; ++ = partial hemolysis; + = slight hemolysis; − = no hemolysis.

oxygen-stable hemolysin as well as limit the production of the oxygen-labile one. That both the oxygen-labile and the oxygen-stable hemolysins were produced in the same culture medium, and by a number of these strains, was clearly shown by Todd's method (1938). Two hemolytic zones were seen in a neutralization test consisting of increasing dilutions of anti-streptolysin-O and a minimum hemolytic dose of the filtrates. The first hemolytic zone was produced by the oxygen-labile hemolysin and hemolysis developed rapidly, whereas the second hemolytic zone developed more slowly in those dilutions in which the oxygen-labile hemolysin had been previously neutralized.

Oxygen-labile hemolysin. A good yield of the oxygen-labile hemolysin appeared in as short a time as 12 hr, although maximum titers were usually obtained after 18 hr incubation. Delay in centrifugation and filtration was found to reduce this titer but not the titer of the oxygen-stable hemolysin. Higher titers were obtained when the pH of the filtrates remained unadjusted at pH 5.2 to 5.6, than when brought (using N NaOH)

TABLE 2

*Inactivation of Clostridium septicum and Clostridium chauvoei oxygen-labile hemolysins by oxidation, and their reactivation by reduction**

Treatment of Filtrate	Vol of Filtrate					
	0.50	0.25	0.12	0.06	0.03	0.01
C. septicum strain Amatsi						
Original hemolysin titer	+++	+++	+++	+++	−	−
Hydrogen peroxide oxidation (Howard, 1953)	++	−	−	−	−	−
Sodium thioglycolate reduction	+++	+++	+++	+++	−	−
Iodine oxidation, (Shwachman *et al.*, 1934)	+++	++	−	−	−	−
Hydrogen sulfide gas reduction	+++	+++	+++	++	−	−
C. chauvoei strain Vallée						
Original hemolysin titer	+++	+++	+++	+++	+++	−
Hydrogen peroxide oxidation	++	+	−	−	−	−
Sodium thioglycolate reduction	+++	+++	+++	+++	++	−
Iodine oxidation	+++	++	+	−	−	−
Hydrogen sulfide gas reduction	+++	+++	+++	+++	+	−

* 2 per cent washed sheep erythrocytes were used throughout these experiments.

TABLE 3

*Hemolysis of rabbit and mouse erythrocytes by Clostridium septicum and Clostridium chauvoei oxygen-labile hemolysins**

Filtrate	Erythrocytes	Vol of Filtrate					
		0.50	0.25	0.12	0.06	0.03	0.01
C. septicum							
281	Rabbit	+++	+++	+++	+	−	−
	Mouse	+++	−	−	−	−	−
501	Rabbit	+++	+++	+++	−	−	−
	Mouse	+++	+++	−	−	−	−
C. chauvoei							
Vallée	Rabbit	+++	+++	+++	+++	−	−
	Mouse	+++	+	−	−	−	−
1500	Rabbit	+++	+++	+++	+	−	−
	Mouse	−	−	−	−	−	−

* +++ = complete hemolysis; ++ = partial hemolysis; + = slight hemolysis; − = no hemolysis.

TABLE 4

Serum values obtained with Clostridium septicum and Clostridium chauvoei strains for the oxygen-labile hemolysin

Antitoxic Sera	*C. septicum* Filtrates			
	281	501	Amatsi	Fenten
C. septicum				
Prévot 1000	100	100	100	100
EX 1632	25	20	20	20
EX 928	55	50	60	55
EX 586	25	25	20	25
EX 588	<4	<4	<4	<4
C. chauvoei				
Prévot 46942	55	80	70	80
EX 1664	700	600	600	600
EX 1658	600	600	600	600
EX 1370	25	20	20	20
	C. chauvoei Filtrates			
	8070	Vallée	Chau 2	1500
C. septicum				
Prévot 1000	100	100	100	100
EX 1632	<4	<4	<4	<4
EX 928	20	15	20	20
EX 586	<4	<4	<4	<4
EX 588	<4	<4	<4	<4
C. chauvoei				
Prévot 46942	100	90	100	100
EX 1664	2000	2000	2000	2000
EX 1658	2000	1800	2100	2000
EX 1370	35	40	40	40

nearer to neutrality. Higher titers were also obtained when physiological saline was used instead of phosphate buffers, ranging from pH 5.5 to 7.5 as diluent (table 1).

Complete hemolysis of sheep erythrocytes occurred with the oxygen-labile hemolysin even at room temperature and within a few minutes of setting up the test. The hemolysin was reversibly inactivated by oxidation and reactivated by reduction (table 2). Oxidation was produced by the addition of hydrogen peroxide (Howard, 1953), and treatment with iodine solution (Shwachman *et al.*, 1934). Reactivation of the oxygen-labile hemolysin was successfully obtained by adding sodium hydrosulfite (Neill, 1926), sodium thioglycolate (Howard, 1953), and cysteine hydrochloride (Guillaumie *et al.*, 1949) and also by bubbling H_2S through the oxidized filtrate (Shwachman *et al.*, 1934).

The oxygen-labile hemolysin was neutralized by anti-streptolysin-O serum, as well as by the normal sera of various animals. Among the 12 normal sera of rabbits, guinea pigs, ox, horse, and man, only 4 (2 of 3 rabbit and 2 of 3 guinea pig sera) failed to neutralize the hemolysin in dilutions varying from 1:40 to 1:320. Mouse erythrocytes were found to be more resistant to the oxygen-labile hemolysin than rabbit erythrocytes (table 3). The neutralization of the oxygen-labile hemolysin by *C. chauvoei* and *C. septicum* antisera is shown by the data in table 4.

Necrotoxin and the oxygen-stable hemolysin.

TABLE 5

*Parallelism in the necrotoxin and the oxygen-stable hemolysin activities**

Filtrate	Necrotoxin				Oxygen-stable Hemolysin				
	Vol of Filtrate								
	0.50	0.25	0.12	0.06	0.80	0.25	0.12	0.06	0.03
Clostridium septicum strain 281									
Activity, fresh	R	R	R	–	+++	+++	+++	+++	–
Inactivation by H_2O_2†	–	–	–	–	–	–	–	–	–
Activity after 48 hr	R	R	–	–	+++	+++	+++	–	–
Activity after 5 days	–	–	–	–	+++	–	–	–	–
Clostridium chauvoei strain 8070									
Activity, fresh	R	–	–	–	+++	+++	+++	–	–
Inactivation by H_2O_2†	–	–	–	–	++	–	–	–	–
Activity after 48 hr	–	–	–	–	+++	–	–	–	–
Activity after 5 days	–	–	–	–	++	–	–	–	–

* R = positive; +++ = complete hemolysis; ++ = partial hemolysis; + = slight hemolysis; – = no hemolysis; no reaction.

† Inactivation was not restored by reduction.

These frequently failed to appear in serum glucose-broth cultures although at times a good yield of the toxin was obtained. It was, however, clear that its production was more favorably obtained when fresh media were used in smaller quantities and when inocula were predominantly sporulating. Larger quantities of relatively old media consistently failed to show any toxin production.

The oxygen-stable hemolysin as well as the necrotoxin were found to lose at least half their activity within the 48 hr which followed their preparation and storage. Various attempts to check their deterioration failed and their loss was usually complete within 7 to 14 days of storage at 4 C. Unlike the instant hemolysis produced by the oxygen-labile hemolysin, an induction period preceded hemolysis of sheep erythrocytes brought in contact with the oxygen-stable hemolysin. This period extended for many minutes and its length clearly, and easily, distinguished the hemolytic system involved. The oxygen-stable hemolysin was not neutralized by either anti-streptolysin-O or normal sera and was irreversibly inactivated with hydrogen peroxide or iodine. The necrotoxin was similarly inactivated and a constant parallelism appeared between its titer and that of the oxygen-stable hemolysin (table 5). This parallelism was again confirmed by the results of neutralization tests made with the *C. septicum* necrotoxin and its oxygen-stable hemolysin (table 6). Parallelism also appeared between the necrotoxin and the oxygen-stable hemolysin of *C. chauvoei*, but it was not found possible to confirm it with neutralization tests because of the poor yield obtained under many cultural and enviromental conditions with the strains tested.

TABLE 6

Serum values obtained against filtrate of Clostridium septicum strain 281

Antitoxic Sera	α-Toxins		β-Toxins	γ-Toxins	δ-Toxins
	Necrotoxic tests	Oxygen-stable hemolytic tests	Deoxyribonuclease tests	Hyaluonidase tests	Oxygen-labile hemolytic tests
C. septicum					
Prévot 1000	100	100	100	100	100
EX 1632	100	80	20	160	25
EX 928	200	200	40	80	55
EX 586	500	400	20	<4	25
EX 588	500	400	15	<4	<4
C. chauvoei					
Prévot 46942	30	40	20	180	85
EX 1664	<4	<4	20	450	700
EX 1658	<4	<4	45	400	600
EX 1370	<4	<4	10	<4	25

TABLE 7

Serum values obtained with Clostridium septicum and Clostridium chauvoei strains for hyaluronidase

Antitoxic Sera	*C. septicum* Filtrates			
	281	501	Amatsi	Fenten
C. septicum				
Prévot 1000	100	100	100	100
EX 1632	160	150	160	160
EX 928	80	80	80	70
EX 586	<4	<4	<4	<4
EX 588	<4	<4	<4	<4
C. chauvoei				
Prévot 46942	180	170	170	160
EX 1664	450	500	450	500
EX 1658	400	500	400	400
EX 1370	<4	<4	<4	<4
	C. chauvoei Filtrates			
	8070	Vallée	Chau 2	1500
C. septicum				
Prévot 1000	100	100	100	100
EX 1632	160	150	150	140
EX 928	70	70	70	80
EX 586	<4	<4	<4	<4
EX 588	<4	<4	<4	<4
C. chauvoei				
Prévot 46942	160	170	180	180
EX 1664	700	700	700	800
EX 1658	700	700	650	700
EX 1370	<4	<4	<4	<4

Hyaluronidases and deoxyribonucleases. All fresh filtrates examined contained a high titer of hyaluronidase and deoxyribonuclease. However, the concentration of these enzymes bore no relation to each other and their titers dropped irregularly at times. Tables 7 and 8 summarize the results obtained with antihyaluronidase and antideoxyribonuclease assay of sera. As with the oxygen-labile hemolysin, *C. septicum* as well as *C. chauvoei* antitoxic sera neutralized hyaluronidase and deoxyribonuclease of both organisms. It is, however, clear that the hemolysins, the hyaluronidases, and the deoxyribonucleases of

TABLE 8

Serum values obtained with Clostrinium septicum and Clostridium chauvoei strains for deoxyribonuclease

Antitoxic Sera	*C. septicum* Filtrates			
	281	501	Amatsi	Fenten
C. septicum				
Prévot 1000	100	100	100	100
EX 1632	20	20	15	25
EX 928	40	40	40	45
EX 586	20	25	25	25
EX 588	15	20	20	25
C. chauvoei				
Prévot 46942	20	20	20	25
EX 1664	20	25	25	35
EX 1658	45	50	45	60
EX 1370	10	12	10	15
	C. chauvoei Filtrates			
	8070	Vallée	Chau 2	1500
C. septicum				
Prévot 1000	100	100	100	100
EX 1632	35	30	25	30
EX 928	60	40	45	45
EX 586	35	30	25	30
EX 588	25	25	25	25
C. chauvoei				
Prévot 46942	150	120	170	120
EX 1664	250	220	250	200
EX 1658	200	200	220	180
EX 1370	80	80	80	85

both organisms are serologically related although not identical, and that at least 2 hyaluronidases and 2 deoxyribonucleases are shared by their filtrates.

DISCUSSION

All of the strains of *C. septicum* and *C. chauvoei* that were tested produced appreciable quantities of hyaluronidases and deoxyribonucleases and most of them readily produced the oxygen-labile hemolysins. On the other hand, the oxygen-stable hemolysins were not uniformly obtained, and a number of strains repeatedly failed to show any trace of them. McCoy and McClung (1938) considered *C. chauvoei* to be a transitional organism between the toxigenic and the nontoxigenic clostridia. And indeed none of its strains produced enough activity to enable necrotoxin, or oxygen-stable hemolysin, neutralization tests to be made. Many methods were tried to increase the toxin yield and to concentrate the filtrate (as by freeze drying or by ammonium sulfate precipitation), but with only partial success. Mason (1936), when faced with the same difficulty, mixed the concentrated filtrate with adrenalin and probably increased the actual necrotizing effect of *C. chauwoei* toxin when it was intradermally injected into guinea pigs. However, in these experiments no adrenalin was used and efforts were hampered by the rapid deterioration in the toxicity of the fresh filtrates and by the incomplete neutralization of the lesions produced by the concentrated preparations.

Eisenberg (1907) reported the hemolysins of *C. septicum* and *C. chauwoei* as partially oxygen-labile, being easily activated by reduction after many months of storage. This was later confirmed by a number of workers who claimed that the hemolysins and the lethal toxins produced by these two organisms were in fact two different substances (Kojima, 1923; Robertson, 1929; Kerrin, 1934; Karube, 1934; Guillaumie, 1947). And it was not until 1942 that Koerber and Alture-Weber drew attention to the presence of an oxygen-stable hemolysin in *C. septicum* filtrates and subsequently used it, instead of the lethal toxin, as a more convenient method of assaying antitoxic sera. This was later substantiated with Bernheimer's (1944) findings that the oxygen-stable hemolysin was identical with the lethal toxin. Glenny *et al.* (1931) has also shown the lethal toxin of *C. septicum* to be identical with the necrotoxin which these experiments prove to have the same characters as the oxygen-stable hemolysin. And thus, the lethal, necrotic, oxygen-stable hemolysin of *C. septicum*, whose production is favored by lengthy incubation in the presence of normal serum, is specific to this organism, being neutralized by homologous antitoxic sera but not by the *C. chauwoei* antitoxic or by the normal sera. Indications were also found to point to a corresponding identity between the oxygen-stable hemolysin of *C. chauwoei* and its necrotoxin.

In contrast to the late appearance of the oxygen-stable hemolysin, the oxygen-labile hemolysin together with the hyaluronidases and deoxyribonucleases have been shown to appear early, reaching their maximum within the first 18 hr of incubation. The oxygen-labile hemolysins of these organisms are serologically related, but not identical, to each other or to those produced

by streptococci, pneumococci (Cowan, 1934), *Clostridium tetani* (Todd, 1934), *Clostridium histolyticum* (Guillaumie, 1942), and the θ-toxin of *Clostridium welchii* (Todd, 1941). They are reversibly inactivated by oxidation and reactivated by reduction and are neutralized to a relatively high titer with anti-streptolysin-O as well as by the normal sera of a variety of animals. High titers of these oxygen-labile hemolysins showed no necrotoxic activities when injected intradermally into depilated guinea pigs; they may, however, possess some lethal action when injected intravenously into mice. The kinetics of hemolysis of these two types of hemolysins are also distinct. Thus instant hemolysis of sheep erythrocytes occurs with the oxygen-labile hemolysin, whereas an induction period of at least several minutes precedes hemolysis by the oxygen-stable hemolysin. Mouse erythrocytes were more resistant than rabbit erythrocytes and the distinction, reported by Cohen *et al.* (1942) with pneumolysin, Oakley (1943) with *C. welchii* θ-toxin and Howard *et al.* (1953) with *C. histolyticum*, is probably common to all serologically related oxygen-labile hemolysins.

These findings also confirm the work of Warrack *et al.* (1951) who found the deoxyribonuclease of *C. septicum* to be antigenically distinct from its lethal toxin. It was referred to as the β-toxin, the *C. septicum* α-toxin presumably being the lethal, necrotoxic, oxygen-stable hemolysin. The hyaluronidase and the oxygen-labile hemolysin of this organism also appear distinct from each other as well as from the α and β-toxins of this organism. The hyaluronidase could thus be referred to as the γ-, and the oxygen-labile hemolysin as the δ-, toxin of this organism. As to *C. chauwoei*, indications point to a similar parallelism between the necrotoxin and the oxygen-stable hemolysin. The deoxyribonuclease of this organism could be referred to as the β-toxin, the hyaluronidase as the γ-toxin, and the oxygen-labile hemolysin as the δ-toxin. All three were found to be antigenically distinct from each other and to be serologically related, but not identical, to the corresponding toxins produced by *C. septicum*.

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SUMMARY

Clostridium septicum and *Clostridium chauwoei* produce more than one toxic substance. The number and type of these components vary, among other things, with the strain of the organism, the culture medium used, and the length of the incubation period.

The lethal, necrotic, oxygen-stable hemolysin (α-toxin) of *C. septicum* is specific for this organism; it is neutralized by homologous antisera, but not by antisera of *C. chauwoei* or by normal sera. Smaller amounts of substances possessing oxygen-stable hemolytic as well as necrotizing activity were similarly detected in filtrates of *C. chauwoei*.

The deoxyribonucleases of *C. septicum* and *C. chauwoei* (β-toxin) were found distinct from the α-toxins, and from the hyaluronidases (γ-toxins) of these organisms. At least 2 deoxyribonucleases and 2 hyaluronidases are shared by their filtrates.

The oxygen-labile hemolysins (δ-toxins) are similarly related but are not identical. They are readily produced by most strains and are reversibly inactivated by oxidation and reactivated by reduction. They are neutralized by anti-streptolysin-O as well as by normal sera. Mouse erythrocytes were relatively more resistant than rabbit erythrocytes to the hemolytic action of the oxygen-labile hemolysin.

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